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PRINCIPAL INVESTIGATOR: Ting C. Suen, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, New York 10016

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6. AUTHOR(S)
Ting C. Suen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University School of Medicine
New York, New York 10016

E-Mail: suen@env.med.nyu.edu

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13. ABSTRACT (Maximum 200 Words)

A paper entitled "Model of transcriptional regulation of the *BRCA1-NBR2* bi-directional transcriptional unit" has been conditionally accepted by the journal *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*. This manuscript is the first detailed study on transcriptional elements located more than 1kb upstream to the minimal BRCA1 promoter. This paper defines the boundary and established a model of how uni-directional transcription is regulated for the BRCA1-NBR2 bi-directional transcription unit. Interestingly, despite the absence of BRCA1 promoter activity in the mouse myoblast cell line, C2C12, the intronic repressor element of BRCA1 appears to be functional in these cells. A recent study has shown an increase in BRCA1 expression during differentiation of a mouse mammary epithelial cell line HC11, as well as the differentiation of C2C12 myoblasts into myotubes. The highly restricted manner of how BRCA1 is regulated implies its important role in cell differentiation.

The work on isolating repressor proteins that we sought to identify has been delayed due to many non-technical issues (see report). Nevertheless, the most critical experiment is now in progress and exciting results are forthcoming. We look forward to file a more complete report when these data are available.

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Introduction

The goal of this proposed research was to identify transcription factors that repress transcription of the human BRCA1 gene by binding to a specific DNA element that we have previously discovered to be located within the first intron of the BRCA1 gene (1). Technically, we planned to use the 36-bp DNA element as a probe in a biotin-streptavidin system to fish out the binding proteins (2). Briefly, the binding site will be labeled with a biotinylated-nucleotide and a low level of ^{32}P (for monitoring the specific binding). Proteins that bind to the binding site can then be separated by the strong interaction between biotin and streptavidin, followed by washes and elution. Proteins purified in sufficient quantity will be subjected to mass spectrometry analysis (3). Once the identities of these proteins are revealed, degenerate oligonucleotide probes will be designed to clone the corresponding cDNAs. Alternatively, it is possible to obtain these clones by comparing with the human genome database.

Body

Unfortunately, due to unanticipated and frustrating long delay in lab renovation, hiring of new personnel, purchases of equipment, and failure of certain instrument, the beginning of the project was much delayed and we fell behind schedule. Despite these obstacles, we have now collected enough quantities of nuclear extracts from several cell lines, including HeLa cells where we first showed the existence of nuclear proteins binding to the repressor element; MCF7 cells, a breast cancer cell line; and SKOV3 cells, an ovarian cancer cell line. A highly efficient system of biomagnetic separation of proteins (4) has been incorporated into the project. We are now at the critical stage of performing the binding reactions, and we are confident that important results will be obtained in the near future.

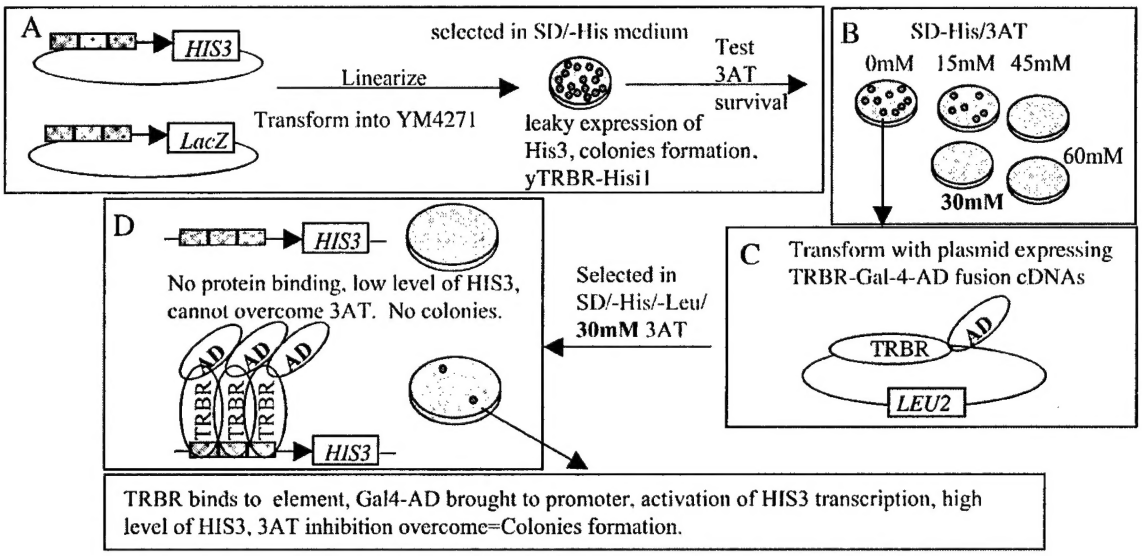
Key Research Accomplishments

Due to the non-technical difficulties we have experienced, we directed a small part of the funding to prepare reagents that will contribute to the success of this project. Although we anticipate success in isolating proteins from our technical objective, it is equally important to have a strategy ready to confirm the functional activity of the proteins that we isolate. We have generated a reporter strain of yeast whose survival will depend on the specific binding of proteins to the specific repressor element. The principle of this yeast one-hybrid system (5) is indicated below:

To confirm the functional activity of TRBR (transcriptional repressor of BRCA1) by a yeast-one-hybrid system

The reporter yeast strain was constructed by cloning multiple copies of the 36-bp transcriptional repressor binding sequence (shaded box in Figure A) in tandem into a yeast integration vector, pHisi1, upstream of a HIS3 minimal promoter driving the expression of HIS3 gene (which encodes the enzyme for synthesizing the essential amino acid, histidine). This vector was linearized and transformed into a yeast host strain YM4271, where a chromosomal integration event could be selected by growing the yeast in minimal essential medium without histidine (Figure A). The newly created reporter yeast strain (yTRBR-Hisi1) was tested for their ability to survive in media containing increasing concentrations of 3-amino-1,2,4-triazole (3-AT) (Figure B), a competitive

inhibitor of the HIS3. The concentration of 3-AT that inhibits the growth of yTRBR-Hisi1 (30mM in this example) is the optimal concentration for testing candidate TRBR.



A candidate TRBR cDNA (that will be identified from the biotin-streptavidin fishing methodology in the proposed work) will be cloned into a yeast expression vector resulting in a fusion of the TRBR cDNA to the activation domain (AD) of the yeast GAL4 transcription factor (Figure C). This yeast expression vector also expresses LEU2 (for leucine synthesis) as a nutritional selection marker. The fusion construct will be transformed into yTRBR-Hisi1 and selected in minimal medium containing the optimal concentration of 3-AT, but lacking both histidine and leucine (double dropout His$^-$Leu$^-$ medium). Only a genuine TRBR-fusion protein that can recognize the binding sites will be brought into close proximity of the HIS3 promoter in yTRBR-Hisi1. Transcription of the HIS3 gene can then be activated by the GAl4-AD to a level where growth inhibition by 3-AT could be overcome, resulting in formation of colonies (Figure D).

Reportable Outcomes

We did not want to stay idle while we were having non-technical difficulties. We therefore directed another small part of the resources to complete a project that was in progress. Although it is not directly related to the proposed work, it does provide further understanding of the repressor element whose interacting proteins were our subject of interest. A manuscript entitled "Model of transcriptional regulation of the *BRCA1-NBR2* bi-directional transcriptional unit" has been conditionally accepted by the journal *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* (6) (Appendices). This manuscript is the first detailed study on transcriptional elements located more than 1kb upstream to the minimal BRCA1 promoter. This work defines the boundary and establishes a model of how uni-directional control of transcription is achieved for the bi-directional transcription unit BRCA1 and its neighboring gene NBR2, which shares a common promoter. In addition, it is very interesting to detect the intronic repressor element of BRCA1 being functional in a mouse myoblast cell line C2C12, where the minimal BRCA1 promoter has been shown previously to be non-functional. A recent

study has shown an increase in BRCA1 expression during differentiation of a mouse mammary epithelial cell line HC11, as well as the differentiation of C2C12 myoblasts into myotubes (7). Understanding the highly restricted manner of how BRCA1 is regulated may also contribute to its role in cell differentiation.

Conclusions

Despite all the delays due to non-technical reasons, we managed to move the project to a critical stage and have implemented a very good strategy for confirming the anticipated data that we will obtain in the near future. We expect to obtain very exciting results in the coming months and look forward to file another more complete report once these data are available.

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Model of transcriptional regulation of the *BRCA1-NBR2* bi-directional transcriptional unit

Ting-Chung Suen[1]*, Moon-shong Tang[1], and Paul E. Goss[2,3]

[1]Nelson Institute of Environmental Medicine, New York University School of Medicine; [2]Breast Cancer Prevention Program, The Toronto Hospital; [3]Breast Group, Department of Medical Oncology, Princess Margaret Hospital, Toronto, Ontario, Canada.

* To whom correspondence should be addressed:

Ting-Chung Suen, Ph.D.

Nelson Institute of Environmental Medicine,

New York University School of Medicine

57 Old Forge Road, Tuxedo, NY 10987

Phone#: (845) 731-3516

FAX#: (845) 351-4510

E-mail: suen@env.med.nyu.edu

Abstract

In contrast to hundreds of mutations found in familial breast and/or ovarian cancers, somatic mutations of *BRCA1* are very rare. However, a high percentage of sporadic breast and ovarian cancers show a reduction in BRCA1 expression, suggesting that defects in transcriptional regulation is a contributing factor. *BRCA1* shares a promoter with its neighboring gene, *NBR2*, which is transcribed in the opposite direction. We have previously shown that transcription of *BRCA1* is negatively regulated by protein factors that interact with a 36-bp segment, located 575-bp into its first intron. We now report the localization of an 18-bp transcriptional repressor element for *NBR2*, which resides 948-bp into its first intron. Binding of nuclear proteins to this 18-bp DNA was detected by electrophoretic mobility shift assays, and it conferred an orientation-dependent functional suppression onto a heterologous thymidine kinase promoter. Combined with our previous studies, a model of transcriptional regulation of the closely aligned *BRCA1-NBR2* bi-directional unit is proposed. A minimal 56-bp DNA region is functional in driving transcription in both directions, while uni-directional control is provided by distinct repressors that bind to sequences located in the first intron of the respective genes.

1. Introduction

BRCA1 is the first breast and ovarian cancer susceptibility gene isolated [1,2], with more than 800 distinct mutations, polymorphisms, and alterations reported throughout its coding sequence in familial breast and ovarian cancer (Breast Cancer Information Core, http://research.nhgri.nih.gov/bic/). BRCA1 has been implicated in important processes such as response to DNA damage, transcriptional regulation, cell cycle checkpoints, and genomic stability, all of which contribute to its tumor suppressor activity [reviewed in 3-7]. The role of *BRCA1* in sporadic cancers is obscure since somatic mutations of the gene are very rare in both sporadic ovarian [8,9] and breast cancers [2,10]. A loss or lowered expression of BRCA1 is frequently found in sporadic breast tumors as compared to surrounding normal tissues [11-14], suggesting that defects in transcriptional regulation is an underlying mechanism. Numerous studies have tried to explain the lowered BRCA1 expression by an epigenetic mechanism such as methylation of the promoter [15-23]. However, in most studies, differences in the methylation pattern between the tumor and its surrounding tissues were only found at a frequency of <15% [24]. Moreover, whether hypermethylation of the promoter is the cause or consequence of loss of gene expression remains uncertain [25-27]. It is therefore very important to understand how transcription of *BRCA1* is controlled. We have previously identified two important determinants of *BRCA1* transcription, and formation of complexes between nuclear proteins and these important DNA elements have been detected by EMSAs [28,29]. Mutations in proteins that regulate transcription of *BRCA1* could result in the loss of BRCA1 expression. This possibility could be one of the reasons why mutations in the *BRCA1* genomic region are not frequently observed in sporadic cancer, since a totally different gene may have been mutated.

BRCA1 and its neighboring gene *NBR2* [30] are aligned in a head-to-head orientation, and separated by only 218-bp. The two genes share a minimal 56-bp promoter region [28] and are transcribed divergently away from each other. Tissue-restricted binding of nuclear proteins to an 18-bp element within the minimal promoter correlates with expression of BRCA1 [28]. Transcription in the *BRCA1* direction is controlled in a negative manner by the binding of nuclear proteins to a 36-bp sequence located 575-bp into its first intron [29]. The existence of a functional repressor activity was also implicated to be located on a 1-kb fragment within the intron 1 of *NBR2* [29]. We now report the fine mapping of this novel repressor element to an 18-bp DNA, located 948-bp into the first intron of *NBR2*. This repressor element was recognized and bound by multiple nuclear proteins from various cell lines and was able to block transcriptional activity in the NBR2 direction. Based on these results and our previous findings, we propose a model of transcriptional regulation for the two closely aligned genes. The two genes share a bi-directional promoter, while uni-directional control is provided by distinct but non-tissue specific repressor proteins interacting with DNA elements located within the first intron of the respective gene.

2. Materials and methods

2.1 Enzymes and reagents

Restriction enzymes, DNA modifying enzymes, including T4 kinase, T4 polymerase, T4 ligase, Klenow fragment, and calf intestinal phosphatase were purchased from Invitrogen (Carlsbad, CA), New England Biolabs (Beverly, MA), Roche Molecular Biochemicals (Indianapolis, IN), or Amersham Biosciences (Piscataway, NJ). Chemicals used for the chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were purchased from Sigma-Aldrich Co. (St.

Louis, MO). Thin Layer Chromatography (TLC) plates were products of Eastman Kodak Co. (Rochester, NY). Cell culture medium and reagents were obtained from Invitrogen (Carlsbad, CA). All isotopes were products from Amersham Biosciences (Piscataway, NJ).

2.2 Plasmids

The plasmid pBluescripts(IIKS) (Stratagene, La Jolla, CA) was used for general subcloning purposes. pMT.IC3 is a plasmid containing multiple cloning sites placed upstream of the CAT gene [31]. The plasmids pNB(*Sst*I-*Spe*I)CAT and pNB(*Sst*I-*Pst*I)CAT have been described in our previous study [29]. To generate deletions from the *Pst*I site, pNB(*Sst*I-*Pst*I)CAT was cut with *Spe*I and *Sma*I, DNA containing the *Spe*I-*Pst*I fragment was gel purified, followed by restriction digest with *Bst*UI, *Nae*I, or *Bse*RI. Both *Bst*UI and *Nae*I generate blunt ends, therefore, the resulting *Spe*I-*Bst*UI and *Spe*I-*Nae*I fragments were ligated back to the *Spe*I- and *Sma*I- restricted pNB(*Sst*I-*Pst*I)CAT vector. The *Bse*RI restricted end was converted to blunt end with T4 polymerase, before the *Spe*I-*Bse*RI fragment could be cloned back into the *Spe*I- and *Sma*I restricted pNB(*Sst*I-*Pst*I)CAT vector. The resulting series of deletion and their positions with respect to the *BRCA1-NBR2* genomic region is shown in Fig. 1. The 130-bp *NaeI-NheI* fragment (Fig. 2A, and schematically shown as a tandem of sub-fragments *h1-h5* in Fig. 2B) that contains the putative repressor element was cloned into the compatible *Sma*I- and *Xba*I-restricted pBluescripts(IIKS). An aliquot of the *Nae*I-*Nhe*I fragment was cut with *Hae*III, followed by ligation with SmaI-restricted pBluescripts(IIKS). Sequencing was performed to determine which of the *Hae*III-*Hae*III fragments were obtained and their orientations (Fragments *I-IV* in Fig. 2B).

2.3 Oligonucleotides

Sequencing primers for either the pMT.IC3 or pBluescript(IIKS) series of plasmids has been described previously [28]. Oligonucleotides were purchased from Invitrogen (Carlsbad, CA), and their sequences are complementary to each other: 5'-GATCCAGAATGGACGCCAA-3' and 5'-GATCTTGGCGTCCATT CTG-3'. After annealing, the sequence of the double-stranded oligonucleotide will be equivalent to that of fragment *h4* in Fig. 2, and its ends are compatible with either a *Bam*HI site or a *Bgl*II site.

To investigate the possible effect of the putative repressor DNA on a heterologous promoter, the double stranded oligonucleotide was ligated to either *Bam*HI- (upstream to the *TK* promoter) or *Bgl*II- (downstream to the *TK* promoter) restricted pBLCAT2 [32], yielding the constructs shown in Fig. 3. pCMVβ (BD Biosciences Clontech, Palo Alto, CA), a plasmid which contains the lacZ gene driven by the cytomegalovirus enhancer [33], was used for monitoring transfection efficiency in all CAT assays. Detailed maps of all plasmids used in this study will be distributed along with the reagents upon request.

2.4 Sequencing

Dideoxy-sequencing of double-stranded plasmids was performed with a T7 polymerase sequencing kit using [α-^{35}S]dATP according to the manufacturer (Amersham Biosciences).

2.5 Cell culture

All cell lines that were used in this study are available from American Type Culture Collection (ATCC, Manassas, VA). This includes HeLa, a human cervical carcinoma cell line; Caco2, a human colon carcinoma cell line; and C2C12, a mouse myoblast cell line. All cell lines were cultured in Dulbecco's modified Eagle's/F12 medium (Invitrogen), supplemented with 10% fetal calf serum, and kept in a humidified, 37°C, 5% CO_2 incubator.

2.6 Transfections and CAT assays

A calcium phosphate precipitation method [34] was used for transfections as modified and described previously [28]. Briefly, cells were split at a predetermined ratio into 100 mm tissue culture dishes (Falcon) the day before transfection. 0.5 μg of pCMVβ and 10 μg of a CAT reporter DNA were added to 0.5 ml of 0.25 M $CaCl_2$. This was followed by dropwise addition of 0.5 ml of 2X BBS buffer (50 mM BES, 280 mM NaCl and 1.5 mM Na_2HPO_4). After 25 minutes at room temperature with gentle mixing, the mixture of DNA-precipitate was added to the cells. Cells were incubated at 37°C for 16 to 20 h, after which they were washed three times with phosphate-buffered saline, re-fed with fresh medium, and returned to the 37°C incubator. Cells were washed and harvested after 20 to 24 hours and several freeze/thaw/vortex cycles were carried out to lyse the cells. One-fifth of the cell lysate was used for the β-galactosidase assay using O-Nitrophenyl-β-D-Galacto-pyranoside (ONPG) as substrate. The results were used to adjust the amount of lysate for the CAT assay. The thin layer chromatography (TLC) method of CAT assays were performed as previously described, where the standard [^{14}C]chloramphenicol was replaced with 1-Deoxy[di-chloro-acetyl-1-^{14}C] chloramphenicol (Amersham Biosciences), and the TLC was exposed to Kodak BIOMAX-MR film at room temperature [28, 29].

2.7 Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described [29]. Nuclear extracts were isolated from different cell lines by means of homogenization under hypotonic condition [35]. DNA fragments were isolated by digesting a plasmid with appropriate restriction enzymes, gel purified, and labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP (depending on the restriction site) by Klenow-fragment. A final volume of 30 μl of reaction mixture was added in the order of H_2O, 10X binding buffer (1X: 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol), 3 μg of poly(dI-dC).poly(dI-dC) (Pharmacia), 10 μg of nuclear extract, an

appropriate amount of unlabeled competitor if desired, and finally, 20,000 c.p.m. of the probe. The mixture was incubated at room temperature for 25 minutes, after which it was loaded onto a 6% native polyacrylamide gel. After electrophoresis, the gel was dried under vacuum in a gel dryer, and exposed to a Kodak BIOMAX-MS film at -80°C.

3. Results

3.1 Localization of a repressor activity in the first intron of the NBR2 gene

We have previously demonstrated the existence of a repressor activity located within a 623-bp region in the first intron of the *NBR2* gene (between the *Pst*I and *Spe*I sites in Fig. 1, nucleotides are numbered the same way as in our previous studies, and transcription of *NBR2* proceeds towards the left) [29]. In order to localize this repressor activity to a smaller region, a series of deletions were made from the *Pst*I site at the 3'-end, and cloned upstream to a CAT reporter gene. Transfections and analyses in HeLa cells showed a significant four- and six-fold increase in CAT activity once deletion was extended to the *Bse*RI site (nucleotide *272*, construct *5*), and further to the SpeI site (nucleotide *624*, construct *6*), respectively. Similar changes in CAT activity were also observed among the various constructs (data not shown) in a mouse myoblasts cell line C2C12, which we have previously shown to lack promoter activity in the *BRCA1* direction [28]. This data localized two repressor activities, one between the *Nae*I and *Bse*RI sites, and another between the *Bse*RI and *Spe*I sites. We chose to characterize the former since it shuts promoter activity almost down to background level.

3.2 Binding of protein factors to the putative repressor element

A 130-bp *Nae*I-*Nhe*I (nucleotides *163-284*) fragment (sequence shown in Fig. 2A, and schematically drawn as fragment *N* in Fig. 2B) containing the putative repressor DNA was

cloned and labeled for EMSAs. Several slower migrating bands representing protein-DNA complexes were observed when the fragment was incubated with nuclear extract isolated from HeLa cells (Fig. 2C, lane *1*). Only those complexes that were clearly competed away by a 100-fold excess of unlabeled *N* itself (lane *2*) were considered to be specific (complexes *N1-N3*). The failure of a 36-bp repressor element (RE) (lane *7*, competitor *R*, located within the first intron of *BRCA1*, positions *2277-2313* in Fig. 1) and a 218-bp *Eco*RI-*Sst*I fragment (lane *8*, competitor *E*, containing the intergenic sequence between *BRCA1* and *NBR2* in Fig. 1) to compete for any of the complexes further confirmed their specificity. To determine the sites of interaction for these protein-DNA complexes, cloned DNA fragments *I-IV*, which contain individual or combinations of sub-fragments *h1-h4* (Fig. 2B) were used as competitors in EMSAs (Fig. 2C, lanes *3-6*). Among fragments *I-IV*, only *III* (lane *5*) competed against the formation of complexes *N1* and *N3*, suggesting these complexes were formed on fragment *III*. On the other hand, none of the four fragments compete for the *N2* complex, suggesting that *N2* was formed on sub-fragment *h5*.

3.3 Localization of specific protein binding to an 18-bp fragment

Since fragment *III* was the only DNA that showed discernable competition against proteins binding to fragment *N*, it was labeled and similarly tested by EMSAs. Judging by the intensity and pattern of the nuclear protein-DNA complexes in Figs. 2C and 2D, complexes *III-1* and *III-3* (lane *2*) are likely to be equivalent to *N1* and *N3*, respectively. An additional complex *III-0* was also detected, and the specificity of these complexes was confirmed by the competitive effect of the unlabeled fragment *III* (lane *3*), but not by fragment *R* (lane *4*). Sub-fragment *h2* is common to both fragments *II* and *III*, yet *II* was ineffective in competing against formation of complexes with *N* (Fig. 2C, compare lane *4* with lane *5*), arguing that the sub-fragment *h4*, which is unique to *III*, was responsible for the formation of these complexes. Indeed, when sub-fragment *h4* was

purified and tested by EMSAs (Fig. 2E), the profile of protein-DNA complexes formation (lane *2*) appears to match that of *III* (Fig. 2D, lane *2*). As expected, these *H*-complexes were strongly and specifically competed by *III*, while both *IV* (lane *4*) and *R* (lane *5*) were unable to compete against their formation. Interestingly, extra bands (*H**) appeared after specific competition with *III* (lane *3*). These could be non-specific or they might be formed by some weaker binding proteins, which became detectable only after the strong binding protein (such as complex *1*) was competed away.

3.4 The 18-bp HaeIII-HaeIII fragment h4 conferred a transcriptional repressor activity onto the heterlogous thymidine kinase promoter

Since fragment *h4* was not able to form all the complexes that were detectable with fragment *N* (complexes labeled with the same number are likely to be formed by the same proteins with the different probes), it was important to determine if it had functional repressor activity. Double-stranded oligonucleotide which contains the same sequence as fragment *h4* (arrow delimited by nucleotides *253* and *236*) was cloned either upstream (Fig. 3A, constructs *2* and *3*) or downstream (constructs *4* and *5*) of a heterologous thymidine kinase promoter (construct *1*, dotted arrow). In its native 5'- to 3'- orientation (i.e. the same alignment as its native direction of transcription, as in constructs *2* and *4*), the fragment was able to suppress the *TK* promoter by 3- to 5-fold in both HeLa and C2C12 cell lines, regardless of whether it is upstream (construct *2*) or downstream (construct *4*) of the promoter. However, when placed in an opposite orientation (constructs *3* and *5*), the fragment was only able to suppress the *TK* promoter by 2-fold in HeLa cells, and marginally in C2C12 cells. Consistent with our previous findings in HeLa cells [29], the RE from the intron 1 of *BRCA1* was able to strongly suppress the *TK* promoter activity regardless of its orientation when placed downstream to the promoter (constructs *6* and *7*).

Interestingly, the *BRCA1* RE was also functional in the C2C12 cells, a cell line which lacks promoter activity in the *BRCA1* direction [28].

3.5 NBR2 repressor is nonfunctional in the BRCA1 direction within the context of its native genomic organization

We have previously shown that the two constructs pBR(*Pst*I-*Sst*I)CAT and pBR(*Spe*I-*Sst*I)CAT (which contain the same fragment but in a reverse orientation as the two constructs pNB(*Sst*I-*Pst*I)CAT and pNB(*Sst*I-*Spe*I)CAT in Fig. 1, respectively) yield similar promoter activity [29], suggesting that the 623-bp *Spe*I-*Pst*I fragment only blocks transcription in the *NBR2* direction. To confirm a directional effect of the now more refined RE, the promoter region of pNB(*Sst*I-*Bse*RI)CAT and pNB(*Nae*I-*Sst*I)CAT (Fig. 1) was reversed to give pBR(*Bse*RI-*Sst*I)CAT and pBR(*Nae*I-*Sst*I)CAT, respectively, thus driving transcription in the *BRCA1* direction. As clearly observed in HeLa cells (Fig. 3 B), pBR(*Nae*I-*Sst*I)CAT was only 10-20% stronger than pBR(*Bse*RI-*Sst*I)CAT, suggesting that the *Nae*I-*Bse*RI region indeed has no repressor activity in the *BRCA1* direction when aligned in its native genomic configuration. In C2C12 cells, both constructs were non-functional, as has previously been shown in this cell line with multiple promoter constructs driving transcription in the *BRCA1* direction [29].

4. Discussion

NBR2 and *BRCA1* are separated by only 218-bp, the first exon and intron of the two genes are therefore immediate upstream of the shared promoter for the opposite genes (Fig. 1). Although the function of NBR2 is not known, and no mutations have been found in its coding sequence [30], an important question of whether these *NBR2* genomic sequences could affect the activity of the *BRCA1* promoter has remained unanswered. We have previously localized a repressor

activity for *NBR2* gene to the 623-bp *Spe*I-*Pst*I region [29]. The deletion analysis showed that there might be two separate repressor regions, one mapped between the *Bse*RI and *Nae*I sites, another between the *Spe*I and *Bse*RI sites (Fig. 1). We chose to characterize the former since this repressor activity was responsible for reducing the promoter activity to almost background level. Transcriptional repressor activity was mapped to the same *Nae*I-*Bse*RI region when the same series of deletion constructs was tested in a mouse myoblast cell line, C2C12 (data not shown). Specific binding of nuclear proteins to the putative repressor *Nae*I-*Nhe*I fragment was detected by EMSAs (Fig. 2C). The clear competitive effects of individual fragments and the similar banding pattern as detected with successive shorter probes (Fig. 2C-2E) strongly suggested that complex *N2* was formed on sub-fragment *h5*, while complexes *N1* and *N3* were formed on the 18-bp fragment *h4* (nucleotide *253-236*). A similar profile of binding (complexes *H1* and *H3*) was indeed detected on fragment *h4* with nuclear extract isolated from HeLa cells (Fig. 2E), and a colon cancer cell line Caco2 (data not shown). Currently, the identities of transcription factors that are responsible for binding to this 18-bp *NBR2* RE are unknown, as no known consensus transcription factor binding sites were found (http://www.gene-regulation. com). Nevertheless, the ability of the 18-bp sequence to suppress the activity of the heterologous *TK* promoter confirmed its repressor function (Fig. 3A). Interestingly, the *NBR2* RE is functional regardless of whether it is upstream or downstream of the *TK* promoter, although an orientation effect was observed. This property distinguishes it from the RE located in the first intron of the *BRCA1* gene, which we have previously shown to function only when placed downstream to the *TK* promoter [ref. 29 and Fig. 3A]. The inability of the 36-bp *BRCA1* RE to compete for protein-DNA complexes formation (fragment *R* in Fig. 2) with all fragments representing the *NBR2* RE further confirmed that different proteins interact with the two distinct REs. Consistent with our

previous results on the repressor activity of the 623-bp *Spe*I-*Pst*I fragment [29], the now refined *Nae*I-*Bse*RI RE clearly had no effect on transcription in the *BRCA1* direction (Fig. 3B).

Recent analyses of the human genome revealed that bi-directional or divergent gene configuration occurs more frequently than previously thought, with a surprisingly high percentage (20% for all gene pairs, and as high as 40% if only DNA-repair genes are considered) of the divergent pairs only separated by <300-bp [36]. Our report here combined with our previous findings [28,29], lead us to propose a model of how uni-directional control may be exerted on this divergently transcribed *BRCA1-NBR2* gene pair (Fig. 4). A 56-bp segment located between *NBR2* and *BRCA1* functions as a promoter for both genes (bi-directional arrow delimited by nucleotides *1377-1432*, numbering of nucleotides are the same as *BRCA1* genomic sequence with Genbank accession no. U37574). Non-tissue specific proteins ITRNB (intronic transcriptional repressor of NBR2, open square) bind to an 18-bp RE, located 948-bp into the first intron of *NBR2* (arrow delimited by nucleotides 236-253), and function to turn off transcription in the *NBR2* direction only. Transcription in the *BRCA1* direction is dependent on the binding of nuclear proteins FRBR (factor required for BRCA1 transcription, open ellipse) to a distinct 18-bp element (open rectangle delimited by nucleotides 1415-1432) within the minimal 56-bp bi-directional promoter. FRBR is absolutely required for BRCA1 transcription to occur, as it is detectable in all cell lines that express BRCA1. In C2C12 cells, the absence of FRBR correlates with a lack of promoter activity only in the *BRCA1* direction, while transcription in the *NBR2* direction is maintained [28]. Non-tissue specific proteins ITRBR (intronic transcriptional repressor of BRCA1, open hexagon) that bind to a 36-bp RE located 575-bp into the first intron of *BRCA1* (arrow delimited by nucleotides 2277-2313) provide negative control of expression

only in the *BRCA1* direction [29]. Interestingly, this negative control is also functional in C2C12 (Fig. 3A).

Absence of BRCA1 function is lethal during mouse development as demonstrated in knock-out mice [37-39]. Overexpression or mis-expression of human BRCA1 has also been shown to block development in mice [40]. Thus, expression of BRCA1 must be tightly regulated. FRBR appears to be responsible for tissue-restricted expression of BRCA1, while the non-tissue specific ITRBR is a good candidate for timely responses of BRCA1 level during various important processes, including development and differentiation [41-44], cell cycle progression [45,46], and response to DNA damaging agents [5,47,48]. Only one published work has tried to study the levels of both NBR2 and BRCA1 in breast cancer cells [49]. It will be interesting to test if the levels of the transcription factors we described here correlate with the expression levels of BRCA1 and NBR2 in those cell lines.

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Fig. 1. Localization of a repressor activity within the intron 1 of *NBR2*. The top line shows a simplified genomic organization for the *NBR2-BRCA1* region. Exons are numbered and shown as open and shaded boxes, and transcription proceeds towards the *right* and *left* sides, for *BRCA1* and *NBR2* genes, respectively. The position and restriction map of a 2.7-kb *Pst*I-*Xba*I fragment is shown below the genomic scheme, and nucleotides in this region are numbered the same way as a *BRCA1* genomic sequence deposited in GenBank (Accession# U37574). The closed rectangle represents a minimal 56-bp bi-directional promoter previous defined by us [28]. The hexagon labeled with "minus" sign represents transcriptional repressors that interact with a 36-bp region (nucleotides 2277-2313) within the first intron of the *BRCA1* gene. The square labeled with a "minus" sign denotes a repressor activity that is located within the 623-bp *Spe*I-*Pst*I region [29]. Numbered leftward pointing arrows (constructs 2-6) correspond to the indicated restriction fragments that were cloned into the empty CAT vector (construct *1*). These constructs were transfected into HeLa cells and activities of the constructs are shown as a relative number to that of construct 6, which was assigned as 100. Experiments were repeated three times and a S.D. <15% was observed.

Fig. 2 Electrophoretic mobility shift assays (EMSA) on the *NBR2* repressor. (A) The sequence of a 130-bp *Nae*I-*Nhe*I fragment covering the putative repressor region as mapped in the deletion experiment, numbered the same way as Genbank sequence accession# U37574. The "C"s at positions *236* and *254* were reported as "N"s in U37574. In this alignment, *NBR2* is transcribed from the opposite strand. Therefore, the direction of transcription for *NBR2* proceeds from the larger to smaller numbered nucleotide. (B) Schematic representation of the *Nhe*I-*Nae*I fragment with positions of *Hae*III restriction sites shown to sub-divide the fragment into 5 sub-fragments,

h1-h5. Subcloned fragments *I-IV* that were used as competitors in EMSAs were sequenced, and found to contain individual or a combination of sub-fragments *h* as shown. (C) Fragment *N* was labeled and incubated with nuclear extract isolated from HeLa cells (lane *1*). The specificity of the slower migrating bands was tested by the addition of a 100-fold excess of unlabeled *N* (lane *2*), fragments *I-IV* (lanes *3-6*, respectively), fragment *R*, a 36-bp DNA which contains a repressor element located in the first intron of *BRCA1* (lane *7*), and fragment *E*, a 218-bp *Eco*RI-*Sst*I fragment (Fig. 1) containing the *BRCA1-NBR2* bi-directional promoter region (lane *8*). Only bands that were clearly competed by the unlabeled *N* were considered specific protein-DNA complexes and are labeled as *N1-N3*. (D) Fragment *III* was labeled (lane *1*) and incubated with HeLa nuclear extract (lanes *2-4*) in the absence (lane *2*) or presence of a 100-fold excess of unlabeled competitor as indicated (lanes *3* and *4*). Bands that were competed away by the unlabeled self-fragment (lane *3*), but not by the non-specific fragment *R* (lane *4*), were considered specific, and labeled as *III-0*, *III-1*, and *III-3*. The band labeled with an asterisk is considered to be a non-specific protein-DNA complex since it was competed away by the non-specific competitor (lane 4), but not by the specific competitor (lane 3) . (E) Fragment *III* was single end-labeled at the 3'-end, cut with *Hae*III, and the resulting fragment *h4* was gel purified (lane *1*) and subjected to an EMSA with nuclear extract isolated from HeLa cells (lanes *2-5*), as in the previous experiments. Again, 100-fold of unlabeled competitors were added as indicated (lanes *3-5*). Judging from the patterns and intensities of the bands, complexes labeled with the same number (e.g. *N1*, *III-1*, and *H1*) likely represent the interaction of the same protein with the successive shortened probes. The band labeled as *H** could either be non-specific, or they might be formed specifically by some weaker binding proteins, which became detectable only after the strong binding protein (such as complex *1*) was specifically competed away (lane 3).

Fig. 3 The 18-bp *NBR2* intronic fragment is sufficient for a repressor activity. (A) a double-stranded oligonucleotide which is equivalent to fragment *h4* in Figs. 2A and 2B (shown as bold arrow, marked from *253* to *236* to indicate the native direction of *NBR2* transcription), was cloned either upstream (constructs *2* and *3*) or downstream (constructs *4* and *5*) of the cytomegalovirus thymidine kinase promoter (construct *1*, dotted arrow). As for control, the 36-bp repressor element from the intron 1 of *BRCA1* was cloned downstream to the *TK* promoter (constructs *6* and *7*), as used in our previous study [29]. These constructs were transfected into both HeLa and C2C12 cell lines and assayed for CAT activities. The CAT data is a typical result from an experiment using HeLa cells. The relative CAT activities in both HeLa and C2C12 cells were shown as the means of three independent experiments, with S.D.<15%. Activity of the *TK* promoter was assigned as 100 for comparison. [B] To determine if the *NBR2* repressor element can function to suppress transcription in the *BRCA1* direction, the promoter region in the two constructs pNB(*Sst*I-*Bse*RI)CAT and pNB(*Sst*I-*Nae*I)CAT (Fig. 1) were reversed to produce the constructs pBR(*Bse*RI-*Sst*I)CAT (lanes 1) and pBR(*Nae*I-*Sst*I)CAT (lanes 2), respectively. The two constructs were transfected into both HeLa and C2C12 cell lines, and a typical result of CAT assay is shown. Activities of constructs are not shown since the relative activity are within 20% of each other for the two constructs in HeLa cells, while they are non-functional in C2C12 cells.

FIG. 4. **Model of transcriptional regulation for the divergently transcribed NBR2-BRCA1 unit.** A 56-bp segment located between *NBR2* and *BRCA1* functions as a promoter for both genes (bi-directional arrow, nucleotides *1377-1432*). Non-tissue specific proteins ITRNB (intronic transcriptional repressor of *NBR2*) bind to an 18-bp RE, located 948-bp into the first

intron of *NBR2* (arrow delimited by nucleotides *236-253*) function to turn off transcription in the *NBR2* direction (leftward) only. Transcription in the *BRCA1* direction (rightward) is dependent on the binding of nuclear proteins FRBR (factor required for *BRCA1* transcription) to a distinct 18-bp element (open rectangle delimited by nucleotides *1415-1432*) within the minimal 56-bp bi-directional promoter. FRBR is absolutely required for *BRCA1* transcription to occur, as it is detectable in all cell lines that express BRCA1. In C2C12 cells, the absence of FRBR correlates with a lack of promoter activity only in the *BRCA1* direction, while transcription in the *NBR2* direction is maintained [28]. Non-tissue specific proteins (ITRBR, intronic transcriptional repressor of *BRCA1*) that bind to a 36-bp RE located 575-bp into the first intron of *BRCA1* (nucleotides *2277-2313*) provide negative control of expression only in the *BRCA1* direction [29]. Based on this work and our previous published data, this model is applicable to cells representing various tissues, including breast, colon, cervix, and ovary [28, 29, and unpublished data].

Fig. 1

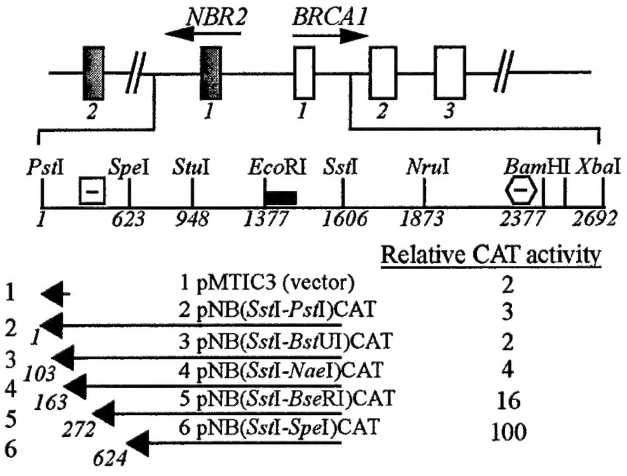

NBR2
BRCA1
2
1
1
2
3
PstI
SpeI
StuI
EcoRI
SstI
NruI
BamHI
XbaI
1
623
948
1377
1606
1873
2377
2692
Relative CAT activity
1 pMTIC3 (vector) 2
2 pNB(SstI-PstI)CAT 3
3 pNB(SstI-BstUI)CAT 2
4 pNB(SstI-NaeI)CAT 4
5 pNB(SstI-BseRI)CAT 16
6 pNB(SstI-SpeI)CAT 100
1
2
3
4
5
6
1
103
163
272
624

Fig. 2

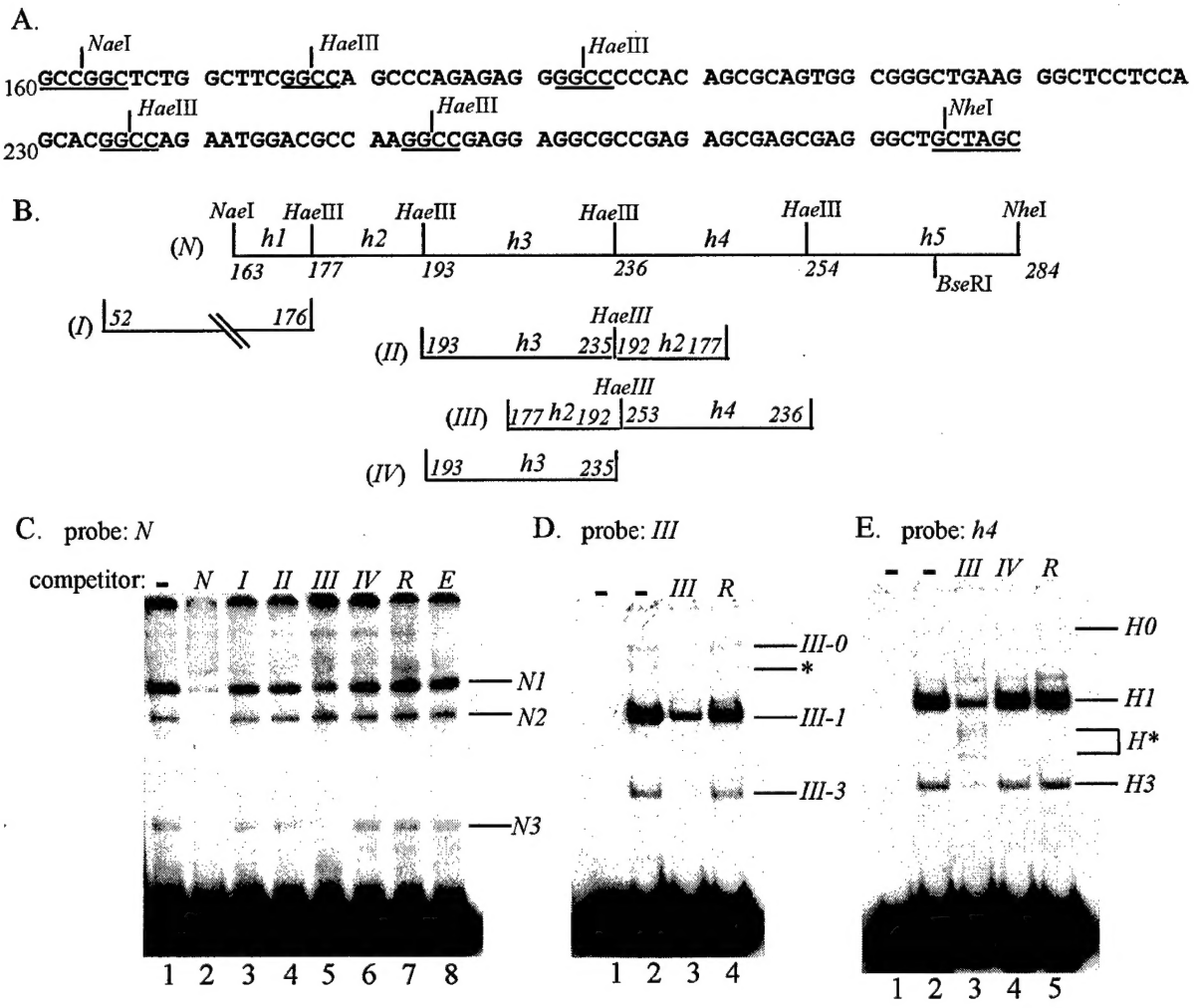

A.
NaeI
HaeIII
HaeIII
HaeIII
160 GCCGGCTCTG GCTTCGGCCA GCCCAGAGAG GGGCCCCCAC AGCGCAGTGG CGGGCTGAAG GGCTCCTCCA
230 GCACGGCCAG AATGGACGCC AAGGCCGAGG AGGCGCCGAG AGCGAGCGAG GGCTGCTAGC
NheI
B.
NaeI HaeIII HaeIII HaeIII HaeIII NheI
(N) h1 h2 h3 h4 h5
163 177 193 236 254 BseRI 284
(I) 52 176
HaeIII
(II) 193 h3 235 192 h2 177
HaeIII
(III) 177 h2 192 253 h4 236
(IV) 193 h3 235
C. probe: N
competitor: - N I II III IV R E
N1
N2
N3
1 2 3 4 5 6 7 8
D. probe: III
- - III R
III-0
*
III-1
III-3
1 2 3 4
E. probe: h4
- - III IV R
H0
H1
H*
H3
1 2 3 4 5

Fig. 3

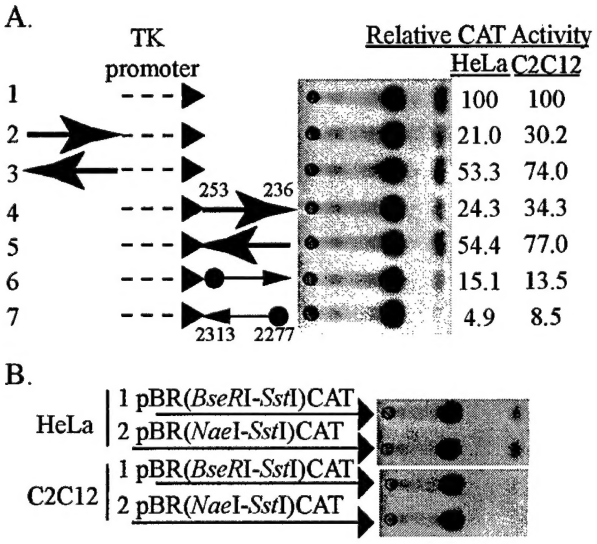
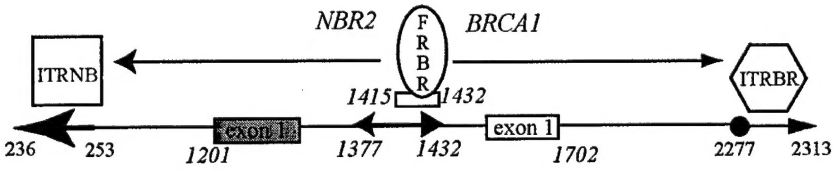

A.
TK
promoter
Relative CAT Activity
HeLa C2C12
1
2
3
4
5
6
7
253
236
2313
2277
100 100
21.0 30.2
53.3 74.0
24.3 34.3
54.4 77.0
15.1 13.5
4.9 8.5
B.
HeLa
1 pBR(BseRI-SstI)CAT
2 pBR(NaeI-SstI)CAT
C2C12
1 pBR(BseRI-SstI)CAT
2 pBR(NaeI-SstI)CAT

Fig. 4


NBR2
FRBR
BRCA1
ITRNB
ITRBR
1415
1432
exon 1
236
253
1201
1377
1432
1702
2277
2313